

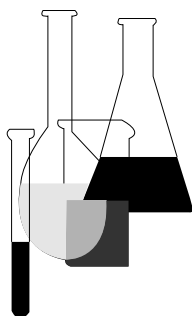


Fate, Transport and Transformation Test Guidelines

OPPTS 835.3400

Anaerobic

Biodegradability of Organic Chemicals



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202-512-1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202-512-0132 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from EPA's World Wide Web site (<http://www.epa.gov/epahome/research.htm>) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines."

OPPTS 835.3400 Anaerobic biodegradability of organic chemicals.

(a) **Scope—(1) Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 796.3140 Anaerobic Biodegradability of Organic Chemicals.

(b) **Introduction—(1) Purpose.** (i) This guideline has been developed for screening for anaerobic biodegradability of organic compounds. A high biodegradability result in this test provides evidence that the test substance will be biodegradable in sewage-treatment plant anaerobic digestors and in many natural anaerobic environments such as swamps, flooded soils, and surface water sediments.

(ii) On the contrary, a low biodegradation result may have other causes than poor biodegradability of the test substance. Inhibition of the microbial inoculum by the substance at the test concentration may be observed. In such cases further work is needed to assess the anaerobic biodegradability and to determine the concentrations at which toxic effects are evident. An estimate of the expected environmental concentration will help to put toxic effects into perspective.

(2) **Principle of the test method.** (i) This portion of this guideline is based on the biodegradability methods referenced under paragraph (e) of this guideline.

(ii) A chemically defined anaerobic medium, containing resazurin as an oxidation reduction indicator and 10 percent (v/v) primary anaerobic digester sludge from a waste treatment plant, is dispensed in 100-mL portions into 160-mL capacity serum bottles. Selected bottles are supplemented with test substance at a concentration equivalent to 50 mg/L as organic carbon. Gas production is measured with a pressure transducer. The extent of biodegradation is determined by comparing gas production from blank control bottles and bottles containing the test substance.

(iii) The average cumulative gas production ($\text{CH}_4 + \text{CO}_2$) is reported, in milliliters, for blank controls, solvent controls, test substances, and any reference compounds. Also reported is the percent of theoretical anaerobic biodegradation at test completion or 56 days, whichever comes first, and the standard deviation between replicate bottles.

(3) **Prerequisites.** The total organic carbon content of the test material should be calculated or, if this is not possible, analyzed, to enable the theoretical yield of $\text{CH}_4 + \text{CO}_2$ to be calculated.

(4) **Guidance information.** (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to a “pass level”.

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(5) **Reference substances.** In some cases, when investigating a substance, reference substances may be useful and an inventory of suitable reference substances needs to be identified. The use of a reference substance is desirable in order to check the activity of the inoculum; ethanol may be used for this purpose. The ethanol must exhibit anaerobic biodegradation (as gas production) greater than 50 percent of the theoretical maximum within 56 days, otherwise the test is regarded as invalid and must be repeated using an inoculum from a different source.

(6) **Applicability.** The method is only applicable to those organic test substances which, at the concentration used in the test, are not inhibitory to bacteria.

(7) **Reproducibility.** The reproducibility of the method has not yet been determined; however, it is believed to be appropriate for a screening test.

(8) **Sensitivity.** The sensitivity of the method is largely determined by the necessity to compare gas production in test substance bottles with gas production in blank control bottles. The method suggests the use of test substances at a concentration of 50 mg/L as organic carbon. This concentration will produce a maximum of 10.5 mL of $\text{CH}_4 + \text{CO}_2$ at 35 °C. Actual measured gas production will be less due to incomplete conversion of all the organic carbon into CH_4 and CO_2 and the extent to which the CO_2 and CH_4 remain solubilized in the aqueous phase. The use of test substance at 50 mg/L as organic carbon represents a compromise between the need to maximize gas production and thus the sensitivity of the test, and the need to minimize the possibility of toxicity to the microbial population.

(c) **Test procedures—(1) Preparations—(i) Required apparatus.** (A) If gas production is measured with a pressure transducer, apparatus such as a 20-gauge syringe needle attached by means of an inert capillary tube to a 3-way valve (Hamilton Mininert valve 3-FLM-IX or equivalent) fitted to a pressure transducer (Unimeasure 100–500 Ω /2mA pressure transducer or equivalent) and an appropriate ohmmeter (e.g. Digitec Model 2120).

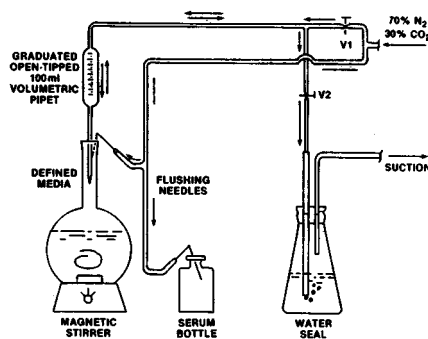
(B) If gas production is measured with a syringe, apparatus such as a 20-mL capacity gas-tight glass syringe fitted with a 20-gauge syringe needle.

(C) If CH_4 and CO_2 are quantified using an analytical procedure specific for these gases, apparatus needed to carry out that analysis, such as a gas chromatograph fitted with a suitable detector.

(D) An incubator sufficient to store the test bottles at $35 \pm 1^\circ\text{C}$ for the duration of the test.

(E) Apparatus suitable for the maintenance of anaerobic conditions during medium preparation and inoculation, such as that shown in the following Figure 1.

FIGURE 1—SCHEMATIC DIAGRAM OF APPARATUS SUITABLE FOR MAINTENANCE OF ANAEROBIC CONDITIONS



(F) A supply of 160-mL capacity serum bottles with butyl rubber stoppers.

(ii) **Nutrient medium**—(A) **Stock solutions**. (1) S-1: Prepare a solution in distilled water containing resazurin at 0.5 g/L.

(2) S-2: Dissolve 20 g ammonium monohydrogen phosphate (NH_4)₂HPO₄ and 100 g of ammonium chloride, NH_4Cl , in distilled water and dilute to 1 L.

(3) S-3: Dissolve 18 g calcium chloride, CaCl_2 , 180 g magnesium chloride hexahydrate, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 130 g potassium chloride, KCl , 2 g manganous chloride tetrahydrate, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3 g cobaltous chloride tetrahydrate, $\text{CoCl}_2 \cdot 4\text{H}_2\text{O}$, 0.6 g boric acid, H_3BO_3 , 0.23 g cupric chloride, CuCl_2 , 1.0 g sodium molybdate dihydrate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.2 g zinc chloride, ZnCl_2 , in distilled water and dilute to 1 L.

(4) S-4: Dissolve 368 g ferrous chloride tetrahydrate, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, in distilled water and dilute to 1 L.

(5) S-5: Dissolve 50 g hydrated sodium sulfide, $\text{Na}_2\text{S}\cdot 9_2\text{O}$ in distilled water and dilute to 1 L.

(B) **Reagents.** Sodium bicarbonate, NaHCO_3 .

(iii) **Inoculum.** (A) The inoculum should consist of sludge from an anaerobic sludge digester. It is recommended that well-mixed primary sludge from a digester with a sludge retention time of 15 to 25 days be used. At the time of collection the sludge should be sieved through a 2-mm mesh screen.

(B) Most sludges can be stored for up to 1 to 2 weeks at 4 °C if necessary, but it is recommended that fresh sludge be used.

(C) Care should be taken to minimize exposure of the sludge to oxygen during collection, handling, and storage.

(2) **Procedure**—(i) **Inoculated medium.** (A) Prereduced medium is prepared by adding 8 mL of stock solution S-1, 8 mL of S-2, and 40 mL of S-3 to approximately 3,500 mL of deionized water in a 4-L Florence or Erlenmeyer flask. This medium is heated to a boil, while being stirred with a magnetic stir bar and sparged with oxygen-free nitrogen. Oxygen-free nitrogen is obtained by passing nitrogen gas through a quartz cylinder filled with copper filings heated to 600 °C. Alternatively, commercial oxygen-free nitrogen may be used.

(B) The flask containing the medium is placed in an ice bath and oxygen-free CO_2 is introduced into the stream of oxygen-free nitrogen to a concentration in the gas stream of 30 percent (v/v).

(C) When the medium has cooled to 35 °C, the flask is removed from the ice bath and the following components are added: 4 mL of solution S-4, 40 mL of solution S-5, 10.56 g sodium bicarbonate, and 400 mL of sludge inoculum. The final volume should be approximately 4 L.

(ii) **Filling test bottles.** (A) Portions of the inoculated medium (100-mL) are transferred anaerobically into serum bottles with a total capacity of 160 mL. Referring to Figure 1 under paragraph (c)(1)(i)(E) of this guideline, V_1 and V_2 are valves that are used to control the transfer of medium to the serum bottles. Inoculated medium is drawn into the pipet by suction, the pipet is moved, and the tip inserted into a serum bottle. During these processes, the serum bottle and neck of the medium flask are continually sparged with the oxygen-free mixture of nitrogen and carbon dioxide.

(B) The medium in the pipet is discharged into the serum bottle.

(C) A new butyl rubber serum-bottle stopper is inserted into the neck of the bottle while the needle used to sparge the contents with the N₂/CO₂ mixture is removed.

(iii) **Test and reference chemicals.** (A) Test and reference chemicals are added to serum bottles to yield a final concentration of 50 mg/L as organic carbon. These chemicals may be added to the bottles prior to the addition of inoculated medium or following addition, depending on the nature of the test or reference substance and whether or not it must be added to the bottles dissolved in a volatile solvent.

(B) Test or reference chemicals with sufficiently high water solubility may be added to test bottles from a neutralized stock solution. The stock solution should be prepared so that a minimal volume is needed to yield 50 mg/L as organic carbon in the medium.

(C) Suitable liquid test or reference chemicals may be added directly by injection from a calibrated syringe.

(D) Test or reference chemicals with relatively low water solubility may be added to test bottles by a direct addition of weighed amounts or by using an organic solvent to dissolve the chemical before addition to test bottles. Direct addition is recommended. If a volatile organic solvent is used, a suitable procedure is to dissolve the chemical in the solvent, pipet an appropriate amount into the bottle, allow the solvent to evaporate, and then add the inoculated medium. Diethyl ether is a suitable solvent for many organics, but it must be completely removed from the bottle because it will adversely affect methanogenesis. If an organic solvent is used without removal of the solvent before the test, the solvent must neither significantly inhibit nor contribute to apparent gas production. Acetonitrile, dioxane, and pyridine have been found acceptable for this purpose.

(E) Bottles containing inoculated medium but no test or reference chemical are employed in each test. These are the blank controls.

(F) If an organic solvent is used to add chemical to test bottles without evaporation of the solvent before the test, bottles containing inoculated medium and an equivalent amount of the organic solvent, but no test or reference chemical, must be employed for each solvent used. These are the solvent controls.

(G) The substance bottles, blank controls, and solvent controls should be prepared in triplicate.

(iv) **Incubation.** (A) At the start of the incubation, pressure in each bottle must be released.

(B) Bottles are incubated in the dark at 35 ± 1 °C for 8 weeks or until biodegradation is complete. Bottles containing oxidized (pink) resazurin should be discarded.

(3) **Analytical measurements.** (i) A sufficient number of measurements of gas pressure or gas volume should be made to establish the pattern of gas production with time. Measurements are generally made weekly for up to 8 weeks. The frequency of measurements may be varied by the investigator as deemed appropriate to match the degradation rate of the chemical.

(ii) Gas production is measured for each bottle using a pressure transducer, syringe, or other suitable apparatus.

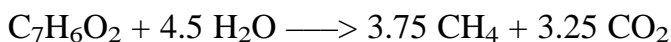
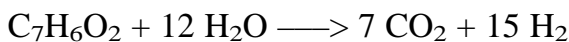
(iii) The use of a pressure transducer is recommended. The ohmmeter should be calibrated by injecting known volumes of gas into bottles containing medium and a standard curve of gas volume vs. meter reading plotted. Excess pressure should be vented after each measurement so that all bottles will have the same internal pressure following each measurement time.

(iv) If a syringe is used to measure gas volume, the following procedure is recommended. The syringe is flushed with 30 percent (v/v) CO₂ in oxygen-free nitrogen. The syringe is held in a horizontal position during the measurement, taking care to keep the needle within the gas space of the serum bottle. Gas production is determined by allowing the syringe plunger to move freely to equalize the vessel and atmospheric pressure.

(v) CH₄ and CO₂ may be determined using analytical methods suitable for the detection and quantification of those compounds.

(d) **Data and reporting—(1) Treatment of results.** (i) Cumulative average gas volume from the anaerobic biodegradation of test or reference substances is calculated by subtracting the cumulative average gas volume production for triplicate blank controls (or solvent controls, if an organic solvent was included) from the average value for triplicate test or reference substance bottles at the same incubation time. The percent of theoretical gas volume produced is calculated by dividing cumulative average gas volume from test or reference chemical by the theoretical maximum gas production and multiplying by 100.

(ii) The maximum CH₄ + CO₂ production theoretically obtainable from an organic chemical in this test is 10.5 mL, if the starting concentration is 50 mg/L as organic carbon. This can be calculated as shown below, using benzoic acid (C₇H₆O₂) as an example.



At a concentration of 50 mg/L as organic carbon in a 100 mL aqueous phase at 35 °C, the maximum volume of gas produced is calculated as follows:

At 50 mg/L as organic carbon in a 100 mL there is 7.27 mg benzoic acid.

$$7.27 \text{ mg benzoic acid} = 0.0595 \text{ mmol}$$

$$0.0595 \text{ mmol} \times 3.75 = 0.2232 \text{ mmol CH}_4$$

$$0.0595 \times 3.25 = 0.1934 \text{ mmol CO}_2$$

$$0.2232 + 0.1934 = 0.4167 \text{ mmol total gas production.}$$

At 35 °C and atmospheric pressure, one mole of gas occupies approximately 25.25 L, thus 0.4167 mmol will occupy 10.5 mL.

(iv) Therefore, any test compound added at a concentration that provides 5 mg of organic carbon to the test bottle will have a theoretical maximum gas production of 10.5 mL.

(2) **Test report.** The following must be reported:

(i) Information on the inoculum including information on the source, retention time, percent volatile solids, date of collection, storage, handling and adaptation possibilities (i.e. information on the possibility that the inoculum was exposed to the test chemical or related chemicals before the test). Retention time and percent volatile solids of the sludge can usually be obtained from the treatment plant operator.

(ii) Average cumulative gas production in milliliters from blank control bottles, solvent control bottles, test substance bottles, and reference compound bottles at each measurement time.

(iii) Percent of theoretical anaerobic biodegradation for each test substance and reference compound at each measurement time.

(iv) The standard deviation for each replicate set of bottles at the final measurement time.

(v) A plot of the percent of theoretical anaerobic biodegradation vs. time for each test substance and reference compound.

(vi) A description of any deviation from this test guideline, such as variations in the medium or the concentration of test substance, test conditions, or analytical techniques.

(e) **References.** The following references should be consulted for additional background material on this test guideline.

- (1) Environmental Protection Agency. J.B. Healy and L.Y. Young, *Methanogenic biodegradation of aromatic compounds*. Workshop on microbial degradation of pollutants in marine environments. EPA Report 600/9-79-012. Gulf Breeze, Florida (1978).
- (2) Gossett, J.M. et al. *Heat treatment of refuse for increasing anaerobic biodegradability*. Stanford University Civil Engineering Technical Report No. 205 (1976).
- (3) Healy, J.B. et al. *Heat treatment of organics for increasing anaerobic biodegradability*. Stanford University Civil Engineering Technical Report No. 222 (1977).
- (4) Healy, J.B. and L.Y. Young. Degradation of simple aromatic compounds under methanogenic conditions. *Abstracts of the Annual Meeting of the American Society for Microbiology*. 13:263 (1977).
- (5) Owen, W.F. et al. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research* 13:485-492 (1979).
- (6) Healy, J.B. and L.Y. Young. Anaerobic biodegradation of eleven aromatic compounds to methane. *Applied and Environmental Microbiology* 38:84-89 (1979).
- (7) Miller, T.C. and M.J. Wolin. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Applied Microbiology* 27:985-987 (1974).